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(54) Title: LIGAND CHELATED PARAMAGNETIC MRI CONTRAST AGENTS

(57) Abstract: Compounds and methods for imaging by MRI tissues having predetermined cellular receptors are described. The imaging agents comprise a receptor-binding ligand covalently bound to a paramagnetic chelate, optionally in combination with compound for enhancing cellular uptake of said agent. Improved MRI is achieved by acquiring MRI signals after period of time sufficient to achieve receptor mediated cellular concentration of the imaging agent.

LIGAND CHELATED PARAMAGNETIC MRI CONTRAST AGENTS

Field of the Invention

The present invention relates to MRI contrast agents. More particularly, the invention is directed to MRI enhancing compositions comprising a paramagnetic chelate linked to a ligand capable of specific binding to a cellular receptor in tissues targeted for MRI and methods for their use.

Background and Summary of the Invention

For more than a century, researchers have been treating brain specimens with chemicals to study brain microanatomy and visualize the previously invisible. The first method for visualizing and quantifying receptors was a process of microscopy called histology. Chemicals, dyes, and stains were used to react with tissue to cause an area of interest in the cell to become visible. Currently, a commonly used method to examine the microanatomy of the brain is through a process called immunohistochemistry. This process uses the principles of immunology to target certain antigens of interest (Appel NM (1997) *Annals NY Academy of Sciences 754:* 155-62). The only requirements for this assay are the availability of an antibody directed toward the antigen of interest and a means of detecting the antigen-antibody complex in tissue.

Another commonly used method for localizing receptors in the brain is by receptor autoradiography. Tissues are exposed to a radiochemical-bound neurotransmitter or other ligand to bind a specific receptor. The membranes are later exposed to photographic film to determine receptor distribution. Prior uses of this assay exhibited limited resolution and difficulty identifying neuroterminal characteristics (Appel NM (1997) *Annals NY Academy of Sciences 754:* 155-62).

Positron Emission Tomography (PET) has also been used to visualize in vivo receptors by employing radiopharmaceuticals (^{11}C, ^{13}N) that are specific for receptors. They do not cause a pharmacological response after binding since only a small amount of the radiopharmaceutical is needed (Raichele, 1998). Another method of imaging which has become popular recently is called single photon emission computerized tomography (SPECT). The increased availability of radiochemicals and

longer duration protocols has led to increased usage of this method (Lever JR, 11gin N, Musachio JL, Scheffel U, Firley PA, Flesher JE-,, Natarajan TK, Wagner HN, and Frost J&J (1998) *Synapse 29:* 172-82). SPECT has been used to visualize many different receptors such as those for dopamine, acetylcholine, opioid, and seretonin (Lever JR, 11gin N, Musachio JL, Scheffel U, Firley PA, Flesher JE-,, Natarajan TK, Wagner HN, and Frost J&J (1998) *Synapse 29:* 172-82.; Madras BK, Jones AG, Mahmood A, Zimmerman RE, Garada B, Holman BL, Davison A, Blundell P, and Meltzer PC (1996) *Synapse 22:* 239-46).

Biochemical receptors in the brain provide favorable physics for MR imaging with MR contrast agents. However, sufficient contrast is needed for visualization when a small amount of agent is used. It is known to visualize receptors using MRI by using ligands with a large molecular weight. Because of the large molecular weight, targets are likely to be limited to those readily accessible from, or in, the vascular system.

A few ligands with Gd^{3+} chelating ability are currently being developed to visualize receptors. Somatostatin and melanocyte stimulating hormone analogues have been developed for the purpose of imaging tumor associated receptors on the surfaces of a tumor (Edwards WB, Fields CG, Anderson CJ, Pajeau TS, Welch MJ, and Fields GB (1994) *J. Med. Chem. 37:* 3749-57., Bard DP, Knight CG, and Page-Thomas DP (1990) *Br. J Cancer 62:* 919-22). The belief is that imaging with these agents will give more insight into the treatment of tumors. The management of the disease would be improved if it were possible to determine whether metastasis had taken place, and to visualize and identify the precise locations. In addition, ligands that chelate Gd^{3+} would be preferable over radiolabeled compounds since repetitive studies could be done with less risk to the patient (Bard DP, Knight CG, and Page-Thomas DP (1990) *Br. J Cancer 62:* 919-22).

A peptide analog of somatostatin, called octreotide, has been created for use as a clinical therapeutic agent because of its growth hormone inhibitory effects. Its potential uses have been enhanced by conjugating it to chelating group for application to SPECT. Also, imaging studies with PET have shown that this reagent is applicable to the measurement of somatostatin receptors (Edwards WB, Fields CG, Anderson CJ, Pajeau TS, Welch MJ, and Fields GB (1994)

J. Med. Chem. 37: 3749-57).

Another known ligand, melanocyte stimulating hormone (MSH), has been used for imaging malignant melanomas. MSH has been conjugated to a chelating group to deliver radiochemicals to the point of interest. It was found that 50% of the injected imaging agent becomes associated with the cells via the receptor. As a result, MSH became a new imaging agent for melanoma. However, no ligand appeared in the brain following intravenous injection, suggesting that the agent cannot cross the blood brain barrier (Bard DP, Knight CG, and Page-Thomas DP (1990) *Br. J Cancer 62:* 919-22).

Methods have been previously used to increase the number of Gd-DTPA molecules attached to ligand backbones. Many studies have employed the use of polylysine to increase the number of gadolinium ions per contrast agent molecule. Using this method, 60 to 70 moieties of Gd-DTPA are linked to a polylysine backbone, and since the efficacy of this molecule has been shown to be very high, the diagnostic dose can be substantially reduced (Schummann-Giampieri G, Schmitt-Willich H, Frenzel T, Press W, and Weinmann H (1991) *Investigative Radiology 26:* 969-74). In terms of relaxivity, studies in mice have shown that a molecule of this type increases the T value per Gd-DTPA residue as much as 30% (Schummann-Giampieri G, Schmitt-Willich H, Frenzel T, Press W, and Weinmann H (1991) *Investigative Radiology 26:* 969-74). Thus, a perceived advantage of using a large number of Gd-DTPAs is that the dosage can be decreased and the margin of safety can be increased for the clinical use of contrast agents. Similarly, dendrimers have been found to have unusually high ion and molecular relaxivities, thereby increasing the contrast of images. Using such molecules, at least 170 gadolinium ions can be bound, and the relaxivity increases 6-fold when compared to Gd-DTPA alone (Wiener EC, Brechbiel MW, Brothers H, Magin RL, Gansow OA, Tomalia DA, and Lauterbur PC (1994) *Magnetic Resonance in Medicine 31:* 1-8). Also, it has been found that the relaxivity of this molecule increases with size, contrary to the trend with other contrast molecules. However, lack of tissue permeability of these branched molecules and their large sizes has, to date, restricted their use to that of vascular imaging agents.

In one aspect of this invention selected ligands covalently linked to a

paramagnetic chelate are provided and used in MRI to provide enhanced MRI signal intensities and image contract. In one specific embodiment a peptide or peptide derivative or analogy, for example, is linked to a paramagnetic chelating at or near the N-terminus. A neurotensin-like peptide can be a peptide having a C-terminus identical or similar to the 5 or 6 C-terminal amino acids in the sequence of the native neurotensin molecule, thus providing the elements necessary for specific binding to the neurotensin receptor (NTRI). The peptide may be branched once, or more than once, at or near the N terminus, to allow the ligand to carry multiple chelating moieties. This provides a means for delivering multiple paramagnetic atoms per peptide molecule, to enhance the MRI signals.

Neurotensin and neurotensin analogs are exemplified ligands, but other compounds, including peptides and other endogenous molecules and derivatives or analogs thereof, known to bind to receptor sites in target tissues can be utilized. For the model described it is known that the C-terminal six residues in neurotensin contain all the elements required for full binding activity. Thus, a neurotensin-like peptide (NT8-13) may be used instead of neurotensin itself, as long as the peptide binds to the neurotensin receptors. Neurotensin-like peptides are disclosed in U.S. Pat. No. 5,760,188. In other embodiments, the Gd^{3+} chelating moiety can be attached to the C-terminal end of the ligand or to one of the side-chains to preserve receptor binding properties.

The chemistry disclosed for creating the ligands contemplates the sequential addition of amino acids to a growing single chain peptide, and thus is readily applicable to any peptide in which the end received by a receptor is a single chain. The chemistry may also be applicable, however, to more complex situations involving multi-chain ligands (such as insulin) provided that a suitable chemistry is available to link the peptide chains properly prior to or after incorporation of the chelating elements.

The paramagnetic chelate bound to the peptide preferably comprises gadolinium, but other known paramagnetic elements can be used. In a preferred embodiment, the compounds of this invention are used for imaging receptor binding in the brain.

Thus, one aspect of this invention is a magnetic resonance imaging

enhancing agent comprising a receptor binding peptide chemically modified for enhanced in vivo stability, and covalently linked to a paramagnetic chelate. In an illustrated embodiment, the paramagnetic chelate comprises gadolinium and the peptide is neurotensin or a neurotensin derivative.

Another aspect of the invention is a method of imaging by MRI tissues having predetermined cellular receptors, said method comprising administering to an animal having said tissues an effective amount of an imaging agent comprising a receptor-binding compound covalently bound to a paramagnetic chelate, and monitoring MRI data for a period of time sufficient for cellular uptake of said imaging agent to obtain enhanced MRI signal intensity. In an illustrated embodiment of this invention the imaging agent is a derivative of a naturally occurring peptide capable of specific binding with said cellular receptors and monitoring takes place for at least sixty minutes. A calcium channel blocker may be co-administered to increase uptake.

An additional aspect of this invention is a composition for use in MRI comprising a receptor-binding compound covalently linked to a paramagnetic chelate, a second compound that increases internalization of the receptor-binding compound, and a pharmaceutically acceptable carrier. In one embodiment the receptor-binding compound may be a peptide or peptide derivative modified for enhanced stability, particularly neurotensin or a neurotensin derivative, and the second compound may be a calcium channel blocker.

Additional features of the present invention will become apparent to those skilled in the art upon consideration of the following detailed description of preferred embodiments exemplifying the best mode of carrying out the invention as presently perceived.

Brief Description of the Drawings

Fig. 1 shows calculations from peptide elutions for each peptide peak and the relationship of each peptide peak to molecular weight.

Fig. 2 shows binding curves showing the ED_{50} for modified peptides, wherein A is the standard, B is 1 DTPA, C is 2 DTPA, D is 4 DTPA, and E is 8 DTPA.

Fig. 3 represents the ability of each modified peptide to bind to the

receptor as represented as a percent of the standard.

Fig. 4 shows a metal chelating assay quantifying the DTPA groups on the modified peptides.

Fig. 5 illustrates the percentage of the theoretical maximum number of DTPA groups associated with each modified peptide.

Figs. 6A and 6B show NT receptor sites; fig. 6A shows published NT receptor sites, whereas fig. 6B shows paramagnetic ligand localization of putative NT binding sites.

Fig. 7 shows changes in MR signal in the presence of 10 nM (open) and 100 nM (cross-hatched) paramagnetic NT ligand in the areas of the rat brain with known high concentrations of NT receptors, including the retrosplenial cortex (RSg/Rsa), pre and para subiculum (Prs), entorhinal cortex (Ent), substantia nigra and ventral tegmental area (SN/VTA).

Figs. 8A-D show binding after time using paramagnetic NT coupled to four Gd chelates; Fig. 8A (15 min.) and Fig. 8B (60 min.) show post-injection of 40 μM gadolinium control, and Fig. 8C (15 min.) and Fig. 8D (60 min) show signal contrast post-injection of 10 μM gadolinium (yellow indicates 50-75% change, red indicates >75% change).

Fig. 9 shows total binding of various NT constructs; NT standard (A), chelate-4 (B), chelate-4-Gd (C), chelate-1-Gd (D), chelate 1 (E).

Fig. 10 similarly shows total binding of various NT constructs; NT standard (A), chelate-4 (B), chelate 2 (C), chelate 1 (D), chelate 1* vs. chelate 1 (E).

Fig. 11 shows inside binding of various NT constructs.

Fig. 12 shows total binding of chelate-4-Gd in the presence of various drugs, DMSO (■), MK886 (▲), NIF (X), CGS (*), Transferin (●), NT control (|).

Fig. 13 shows inside binding of the various drugs used in Fig. 12.

Fig. 14 shows a stability competition assay, wherein various NT constructs were in competition with ^{125}I-NT; NT control (B), chi-NT 8-13 (A), 4-Gd-chi-NT (C).

Detailed Description of the Invention

Magnetic resonance imaging (MRI) uses low radio frequency waves to

penetrate various tissues of the body. These radio waves are reflected as a result of magnetized spins of protons (Hashemi, RH (1997) MRI: The Basics. Baltimore: Williams and Wilkins). If unpaired spinning protons are placed in a magnetic field, they line up according to the field's direction. If a radio-frequency (RF) wave of specific frequency is sent into the tissue, the spins of some the protons will change direction. After the RF pulse is turned off, these protons return to their equilibrium position, and, in doing so, create a signal (Hashemi, RH (1997) MRI: The Basics. Baltimore: Williams and Wilkins). This is the signal that is transformed into an image during MRI.

When an external magnetic field, B_0, is applied, the protons will line themselves up with B_0. However, they do not all line up in the same direction; half of the protons point in the direction opposite B_0. Eventually enough protons line up to cause a net magnetization in the direction of B_0 (Bandettini PA and Wong EC (1997) *Neurosurgery Clinics of North America 8:* 345-71).

If net magnetization versus time were graphed, an exponentially growing curve would result. There is a time constant associated with this curve which is dependent on the kind of the tissue being imaged and the strength of the magnet.

This time constant is designated T_1, and is described as the recovery of magnetization along the axis of the B_0 field (Bandettini PA and Wong EC (1997) *Neurosurgery Clinics of North America 8:* 345-71). Magnetic strength B_0 and T_1 are directly related, and, as a result, a decrease in one corresponds to a decrease in the other. The term "relaxation" means that proton spins are relaxing back to their lowest energy state. There are three relaxation times associated with MRI: T1, T2, and T2*. T1 and T2 are inherent properties of tissues and thus are fixed for that tissue with a constant B_0. T2* has the same property, but, in addition, it also depends on inhomogeneities in the magnetic field (Marthur-De Vre R and Lemort M (1995) *The British Journal of Radiology 68:* 25-47).

When an MRI is conducted, a contrast agent may be used in order to visualize the point of interest. The magnetic susceptibility of a contrast agent is a measure of how magnetized that chemical may become (Hashemi, RH (1997) MRI: The Basics. Baltimore: Williams and Wilkins). Three types of substances, each with different magnetic susceptibilities, are commonly used in MRI: diamagnetic,

paramagnetic, and ferromagnetic.

Paramagnetic substances have unpaired orbital electrons. They become magnetized when B_0 is on and become demagnetized when it is turned off. Their induced magnetic field, M, is in the same direction as B_0 and their presence causes an increase in the effective magnetic field (Hashemi, RH (1997) MRI: The Basics. Baltimore: Williams and Wilkins). The element in the periodic table with the greatest number of unpaired electrons is gadolinium (Gd), a member of the lanthanide group, with seven unpaired electrons. This large number of unpaired electrons makes gadolinium very paramagnetic and therefore commonly used as a contrast agent in MRI.

Since gadolinium ions [Gd(III)] have one of the strongest degrees of proton relaxation enhancement, gadolinium's strong relaxivity allows for a lower number of chelates to be associated with the ligand to produce a sufficient signal intensity for visualization.

Signal intensity of tissue depends on hydrogen-nucleus concentrations, the velocity of protons moving through the area, and tissue relaxation time (T1, T2). Paramagnetic ions increase both TI and T2 times and can therefore raise or lower signal intensity depending on concentration (Brasch RC (I 993) *Journal of Computer Assisted Tomography* 17(Supplement 1): S14-S18). Unlike the iodinated material frequently used for imaging, contrast agents work indirectly through their effects on the relaxation times of neighboring protons.

While other substances can be used, gadolinium is suited to contrast enhancement because of the large number of unpaired electrons it possesses. This is a good property for contrast since the relaxation rate varies with the square of the magnetic moment, and an electron with an unpaired spin produces a moment 700 times that of H^+ (Brasch RC (I 993) *Journal of Computer Assisted Tomography* 17(Supplement 1): S14-S18).

Gadolinium is chelated to improve its biological tolerance. At physiological pH, free Gd(III) forms an insoluble salt that can be deposited and stored in liver, spleen, and skeletal tissue (Brasch RC (I 993) *Journal of Computer Assisted Tomography* 17(Supplement 1): S14-S18). Lethal toxicity levels would be attained if the free ion were administered to humans. Presently, gadolinium cannot covalently

attach to organic molecules, so chelates are used to trap the ion. Chelates work by creating a cage around the Gd(III) by way of ionic bonds and electrostatic interactions. These chelates are used to enhance gadolinium's solubility and facilitate its entry into tissues (Marthur-De Vre R and Lemort M (1995) *The British Journal of Radiology 68:* 225-47). Chelation also assists with clearance of the gadolinium, and mouse models have shown a one order of magnitude increase in the LD_{50} with chelated Gd(III), as opposed to the free ion (Bradley WG, Brant-Zawadzki MN, and Runge VM (1991) *Radiology 181:* 701-9).

The most commonly used MRI enhancing agent is gadolinium-diethylenetriamine pentaacetic acid (Gd-DTPA). This molecule has been found to have the same pharmacokinetics as iodinated x-ray agents (Desreux JF and Barthelemy PP (1988) *Nucl. Med. Biol. 5:* 9-15). DTPA is very water soluble due to its five carboxy (COOH) groups. In addition, every nitrogen carries a pair of electrons to coordinate with lanthanide group members. One disadvantage of chelating gadolinium is the loss of some relaxation enhancement. However, if H_2O molecules interact with the Gd(III) then the Gd (III) remains effective (Tweedle MF, Gaughan GT, Hagan J, Wedeking PW, Sibley P, Wilson LJ, and Lee DW (1998) *Nucl. Med Biol. 15: 31-36*). Since the discovery and production of this first MRI contrast agent, other paramagnetic chelators have been designed and have become commercially available.

Many different designs for Gd(III) chelating groups have been invented and produced in the last few years. One of these is Gd DTPA-BMA, where BMA is for bis methylamide. This is basically a non-ionic form of DTPA that reduces the relaxivity of Gd ions only 1/3 as much as DTPA (Dawson and Blomley, 1994).

Other structures, such as cyclic or macrocyclic chelators, are known. (Wedeking et al, 1992). However, the precise structure of the chelator is not important for the present invention. The 3^+ charge of Gd is balanced by the three carboxy negative charges in DTPA, creating a non-ionic, neutral molecule. Also, as with DTPA-BMA, the relaxivity of the Gd ion is reduced by 1/3 when compared to DTPA (Dawson and Blomley, 1994).

Although the different designs for chelating agents differ in chemical

structure, they are all based on the same general principles, and all possess the same biodistribution and pharmacokinetics (Wedeking et al, 1992). Also, there have been no convincing data to support claims that one chelating agent is clinically more useful than another.

Neurotensin (NT) is a thirteen amino acid peptide (Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH (SEQ ID NO. 1)) first isolated from extracts of bovine hypothalamus during the purification of substance P (Carraway RE and Leeman SE (1973) *J Biol. Chem. 248:* 6854-61). It was named due to its potent hypotensive effects and neural location. It is now established, however, that neurotensin is present in both the central nervous system (CNS) and in peripheral tissues. The major peripheral tissue source of NT is the gastrointestinal tract, where NT is considered a hormone. NT is a member of a family of related peptides all possessing common carboxy-terminal sequences and similar biological effects. NT-like immunoreactivity has been found to be abundant in the rat gastrointestinal tract and widely distributed in the CNS; mostly in the forebrain and limbic system (Carraway RE and Leeman SE (1976) *J. Biol. Chem. 251:* 7045-52). In mammals, NT immunoreactive cells have also been found in the epithelium of the jejunum-ileum where NT is thought to play a role in fat metabolism and regulation (Ferris CF, Armstrong MJ, George JK, Stevens CA, Carraway RE, and Leeman SE (1985) *Endocrinology 116:* 1133-38). Anatomical and pharmacological data of the CNS suggest a close relationship between neurotensin and doparninergic systems. NT has been shown to control the synthesis and release of dopamine, as well as the sensitivity of some dopamine receptors (Quirion R (1983) *Peptides 4:* 609-15.),

Neurotensin produces a large number of physiological and behavioral effects after systemic administration. These effects include: hypotension, hyperglycemia, smooth muscle contraction, and inhibition of gastric secretions (Prange AJ and Nemeroff MA (1982) *Annals NY Academy of Sciences 400:* 368-76). Also, after cerebroventricular injection of NT, the following effects have been observed: sedation, hypothermia, muscle relaxation, reduced food consumption, and changes in locomotor functions (Prange AJ and Nemeroff MA (1982) *Annals NY Academy of Sciences 400:* 368-76). These and other data have suggested that these centrally-specific effects mimic those of various neuroleptics.

The effects of neurotensin observed after its administration result from specific interactions with its receptor, which is present on cell membranes. Binding experiments have indicated the presence of two different neurotensin binding sites (Vincent JP (1995) *Cellular and Molecular Neurobiology 15:* 501-12). One is a high affinity receptor (NTR1) that has been fully characterized, and the other is a low affinity receptor that is less understood (NTR2). The receptors share 60% homology and are structurally similar to the family of G-protein-coupled receptors with seven transmembrane domains connected by intracellular and extracellular loops. (Vincent JP (1995) *Cellular and Molecular Neurobiology 15:* 501-12). Binding studies with iodinated NT fragments indicate that all the binding affinity resides in its carboxy-terminal end, between amino acids 8 and 13 (Vincent JP (1995) *Cellular and Molecular Neurobiology 15:* 501-12). Its amino-terminal end does not bind to the receptor.

Distribution of neurotensin receptors in the rat brain has been determined by autoradiography and immunohistochemistry. In the central nervous system, NT immunoreactive neurons are distributed within the hypothalamus, amygdala, basal forebrain, midbrain tegmentum, and lower brainstem (Boudin H, Pelaprat D, Rostene W. and Beaudet A (1996) *The Journal of Comparative Neurology 373:* 76-89). High amounts of NT-like immunoreactivity have also been found in areas enriched with dopaminergic neurons (substantia nigra and ventral tegemental area) and nerve terminals (neostriatum and nucleus accumbens) (Quirion R, Rowe WB, Lapchak PA, Araujo DM, and Beaudet A (1992) *Annals NY Academy of Sciences 668:* 109-19). Moderate binding areas are found in the striatum and ventral hippocampus (Boudin H, Pelaprat D, Rostene W. and Beaudet A (1996) *The Journal of Comparative Neurology 373:* 76-89). For the examples in this invention, MRI imaging is focused on the previously mentioned high density receptor areas of the brain.

Prior MRI animal research has been limited due to technical problems associated with the movement of the animal in the magnet. Any slight movement can disrupt the image and create false changes in signal intensity. In the present invention, the contrast ligands that have been synthesized were tested in the brain of a rat. This required the use of an awake, motionless animal so that images could be

acquired and clearance rates calculated. For this reason, a specially designed restraint system was used to keep the rat still throughout the experiments (Lahti KM, Ferris CF, Li F, Sotak CH, and King JA (1998) *Journal of Neuroscience Methods 82:* 75-83). The holder is constructed entirely of PLEXIGLAS and plastic so that it can be placed within the magnetic field without disruption. The holder is designed to allow unrestrained respiration. It has been reported by Lahti KM, Ferris CF, Li F, Sotak CH, and King JA (1998) *Journal of Neuroscience Methods 82:* 75-83. that an animal in such a holder does not indicate any discomfort or undue stress.

A method was devised for single-step solid-phase peptide synthesis of peptide ligands containing multiple Gd-chelating moieties. Neurotensin (NT), offers advantages in regard to the availability of receptors and the well-defined dependence of receptor binding on C-terminal functional groups within NT. Since peptide synthesis proceeds from C-terminus to N-terminus, it was a simple matter to construct the NT-like ligand and, while it was still on the resin, to extend its N-terminus with a spacer followed by a variable number of Gd-chelating moieties. Since receptor binding is known to be unaffected by the addition of these groups to the N-terminal portion of NT, the various compounds could be compared without this variable presenting a problem.

Peptides were assembled on a scale of 25 μmol using the Wang resin loaded with Fmoc-Leucine (L) attached through its COOH-group. After removal of the Fmoc-blocking group using piperidine, the exposed amino group was reacted with a 5-fold excess of Fmoc-Isoleucine (1) activated with HBTU, giving the protected dipeptide, Fmoc-IL-resin. After removal of the new Fmoc-group, the appropriate Fmoc amino acids were added successively to construct the peptide SASELYENKPRRPYIL-resin (SEQ ID NO:3) or similar peptide sequences containing the NT-like ligand (KPRRPYIL (residues 6-13 of SEQ ID NO. 1)) at the C-terminus. All of the peptides contained a spacer region (in this case, SASELYEN (SEQ ID NO. 2)) which separated the binding moiety from the N-terminal where the chelates would eventually be attached.

In order to permit the attachment of multiple chelates to the N-terminus, a branched-chain lysine residue was added to the growing N-terminus by reaction with a 5-fold excess of Fmoc-(Fmoc)-Lysine. Upon removal of the Fmoc-

group, this reaction doubled the number of amino groups at the N-terminus of each peptide. By varying the number of times this reaction was performed, batches of resin were prepared which contained 1, 2, 4, 8, 16, and 32 amino groups per peptide chain.

The final step of the synthesis involved the addition of one chelating moiety to each amino group in the growing peptide chain. This was accomplished by reaction of the peptide resin twice with a 5-fold excess of DTPA-dianhydride in the presence of a base catalyst. After removal of excess reagents, the peptides were cleaved from the resins using TFA and then purified to homogeneity by HPLC on m-Bondapak C18. This yielded a series of compounds having a variable but defined number of chelating groups present at the N-terminus of the NT-like ligand. The structure of each compound was verified by amino acid analysis. The molar reactivity of each compound in a NT radioreceptor assay was found to be 30- 100% relative to NT, indicating that binding of the ligand was not greatly hindered by the presence of the chelates. The chelating ability of each compound was determined using radioactive (^{51}Cr) chromic chloride.

Neurotensin was the peptide model used for the above experiments, however, it is understood that other peptides can be modified in the same way. A change in the binding sequence, if it is located at the carboxy terminus, is all that is needed to modify the peptide to make the peptide specific for other receptors. Moreover, the development of a universal branch chain peptide that can chelate multiple gadoliniums allows this approach to be applied regardless of whether the binding site is at the carboxy terminus. Thus, this approach provides a very powerful tool for imaging receptors since it would be conceivable that any molecule that contained the functional group could be used for imaging. This technology could then be expanded to study drug-receptor interactions and determine their biodistribution more accurately.

It is understood that the paramagnetic peptides of this invention can be designed to target a wide variety of receptors within the central nervous system and peripherally. One such use of the paramagnetic peptides of this invention is for visualizing cancer cells in peripheral tissue. For example, NT can be used to visualize various tumors, including prostate, colon, and pancreatic tumors. Also, vasopressin receptors are normally found in gastrointestinal tissue. However,

small cell carcinoma cells are also known to express the vasopressin receptor. A paramagnetic vasopressin-like peptide could be used to detect small cell carcinoma. Increases in signal over time in unexpected locations may indicate the presence of such a cancer. Other examples include bombesin and cholecystokinin. Similarly, steriods and other compounds that interact with cell receptors may be modified to incorporate paramagnetic ligands. Because these paramagnetic compounds are likely internalized, and the signal increases over time, these paramagnetic compounds are useful for visualizing a wide range of receptors throughout the body.

Example 1

Chelated Ligand Synthesis

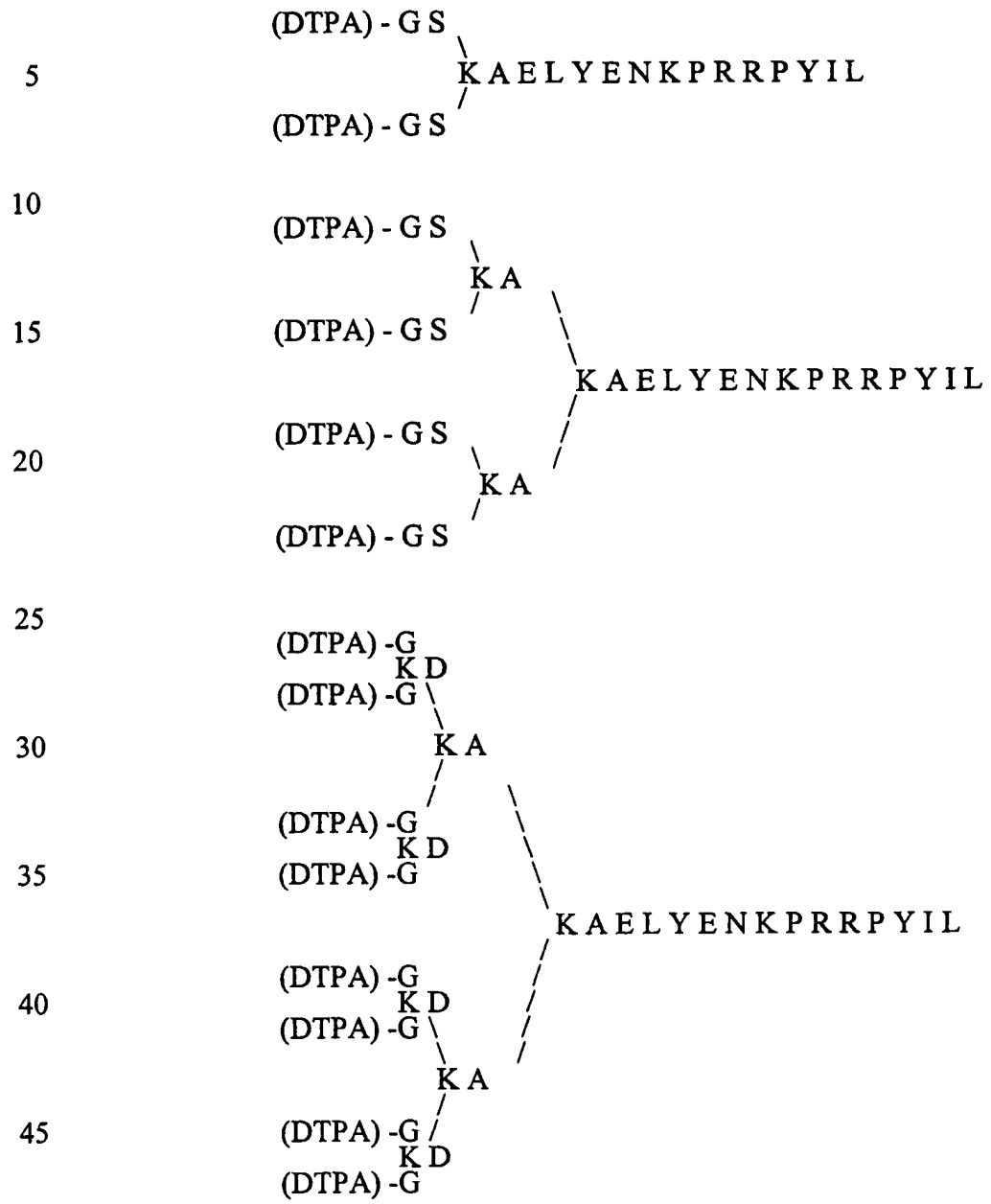
Neurotensin peptides for use in MRI studies were synthesized on a 25 mmol scale using an automated Rainin Symphony synthesizer with WANG resin, Fmoc amino acids, and HBTU activation in the presence of 4-methylmorpholine. Deprotection was accomplished using 20% piperidine for 9 minutes. Cleavage of the peptides from the resin was performed with 86% trifluoroacetic acid, 5% H_2O, 5% anisole, 2% triisopropylsilane, 1% thiophenol, and 1% ethanedithiol. Peptides were precipitated, dissolved in water, and lyophilized. The resulting powders were dissolved in 10 mL of acetonitrile, and water was added to maintain the solution. Chromatography was performed on a 10 x 25 cm column of m-Bondapak C18 (Waters) using a linear gradient (60 min) from 15% acetonitrile to 75% acetonitrile and a flow rate of 6 mL/min. Eluted peptides were identified by their absorbance at 280 nm and their constituent amino acids determined after hydrolysis in 6 N HCl at 150°C for 1.5 hours. Amino acid analysis was performed with the Accutag system using Water's HPLC and computerized Millenium software for quantitation of HPLC identified peaks (Waters). Each peptide gave integral molar ratios of the appropriate constituent amino acids.

After synthesis was complete, but before cleavage from the resin, diethylenetriamine pentaactetic acid (DTPA) was coupled to the amino groups on the peptides. A straight chain peptide was used to create a ligand with a single DTPA group:

(DTPA) - G S K A E L Y E N K P R R P Y I L (SEQ ID NO. 14)

Peptides were branched, to different degrees, at the amino terminal lysine groups to

create two, four, and eight moles of DTPA groups per mole of neurotensin, as shown below:



Two m moles of DTPA (0.8g) plus 2 m moles of 1-hydroxybenzotriazole hydrate (HoBt) and carbodiimide were dissolved in 10 ml DMSO with heating for 60°C. After cooling to 22°C, one-half of the solution was added to the peptide reaction vessel, followed by 2 m moles of diisopropyl-carbodiimide. After 1 hour reaction time, the vessel was drained and the procedure was repeated.

A base catalyst, triethylamine (TEA), was added to the resin before

cleavage in order to activate the amino groups of the peptides. One mole of TEA in 1 ml dimethyl sulfoxide DMSO was added per mole of amino group on the peptide, and the mixture was allowed to react for 5 minutes at room temperature. The reaction vessel was drained and TEA in DMSO was added again. Then, a ten fold excess of DTPA dianhydride, in proportion to the amino groups on the peptide, (suspended in 4 mL DMSO)was added and allowed to react for one hour at room temperature. After the incubation, the mixture was drained from the resin and the reaction was repeated.

Each synthesized peptide was purified by HPLC on m bondapak C18 and was found to elute as a contiguous peak of optical density (280mm). Acid hydrolysates of the peak fractions were found to contain the expected integral molar ratios of amino acids.

A 10 mM gadolinium acetate solution was made by adding gadolinium chloride (Sigma Chemical Co., St. Louis, MO) to 0.4 M sodium acetate at pH 5.5. Chelation of gadolinium to the DTPA groups on the peptides was accomplished by adding a 50 fold excess of gadolinium acetate to 1 mg of the peptide. The total volume was kept at 1 mL. This mixture was incubated for 16 hours at room temperature in a plastic 15 mL screwcap tube.

Following completion of the gadolinium chelating reaction, the peptide was purified on a Sephadex column with a volume of 160 mL. The column was packed with 50% G-25, 50% G-50 resin that was washed once with 5% acetic acid and four times with distilled water prior to its use. The column was equilibrated for 16 hours with 20 mM sodium acetate at pH 5.5.

The gadolinium labeled peptide mixture (1 ml) was mixed with 0.05 ml of a solution of visible dye (phenyl red) and then applied to the column. The column was developed using 20 mM sodium acetate (pH 5.5) at a flow rate of 0.3 mL/min. The first 30 mL was discarded and then 2 mL fractions (70 drops/tube) were collected in 13 x 100 mm plastic tubes.

Fractions collected from the column chromatography were assayed for peptide activity by use of a neurotensin radioimmunoassay (RIA). Dilutions of the fractions were made in order to obtain readable quantities of peptide (1-200 fmol). The RIA reaction volume contained 100 mL (18,000 cpm) of ^{125}I-NT prepared according to Carraway RE and Leeman SE (1976) *J. Biol. Chem. 251:* 7045-52., 100

mL of diluted HC-8 antibody specific for the carboxy terminus of the NT sequence, and 10-50 mL of diluted sample. Phosphate buffered saline gel at pH 7.4 (recipe - Appendix A) was used to bring the total volume to 500 mL. Also, known quantities of unmodified NT were used to check the efficiency of the assay and construct a standard curve.

Reaction mixtures were kept at 4°C for 18 hours. The "bound" (B) and "free") (F) trace NT were separated at 4°C by addition of 1 mL of a 1:4 dilution of charcoal (2.5%)/dextran T-70 (0.25%) into phosphate buffered saline. The tubes were left at 4°C for 10 minutes and then centrifuged for 30 minutes at 3000 rpm. The supernatant (B) was counted with an automatic gamma counter for two minutes and the B/F ratios in the presence of antibody were corrected by a negative control containing no antibody.

Results were graphed according to fmol/mL immunoreactive NT(based on the standard curve) vs. fraction number. The K_{av} was calculated for the earliest peak by the equation $Kav = (V_e - V_o)/(V_t - V_o)$ where V_o is the void of the column (mL), V_t is the volume of the column (mL), and V_e is the volume at sample's peak (mL). Fractions included in the peak of interest were collected in a 50 mL tube and lyophilized.

The K_{av} was also calculated for each peptide peak as a way of comparing the elution behavior (Fig. 1). According to standards of known molecular weight run on the column (data not shown) it was found that peptides elute according to molecular size, with the larger species eluting first. Fig. 1 shows that the peptides synthesized for this project eluted in the correct order according to their calculated molecular weights. The free Gd^{+3} eluted after the peptide-bound Gd^{+3} complexes and was thus, separated from the peptides.

Example 2

Receptor Binding Assay

Receptor binding assays using the probes of Example 1 were performed on a prostate cancer cell line (PC-3) that expresses neurotensin receptor (Seethalakshmi et al., 1997). The cells were grown to 90-95% confluence in a 24 well polystyrene plate. The media was removed from the plate and the wells were washed once with 650 mL of warmed KRB buffer (1 L of KRB Buffer contains 8.5g NaCl,

0.45g KCL, 0.15g MgCl, 0.25g CaCl, 1.5g Hepes, 0.2g $NaHCO_3$, 1g Glucose, and 1g BSA). Cells were incubated at room temperature for three minutes and then the buffer was removed. The reaction mixture added to each well of cells contained 0.8 mL KRB buffer, 0.1 mL of modified peptide at concentrations from 100 pM-100 nM, and 0.1 mL of ^{125}I-NT (100,000 cpm) prepared according to Carraway RE and Leeman SE (1976) *J. Biol. Chem. 251:* 7045-52.

The cells were incubated for 30 minutes in a 37°C water bath. The plate was then transferred to ice and allowed to incubate for 20 minutes. The reaction mixture was aspirated and the wells washed three times with ice cold saline (10 g/L). 0.25 M NaOH/ 0.05% SDS was added to the bottom of the wells and the mixture was incubated in a 37°C water bath for 15 minutes. The cell eluates were transferred to 12x75 mm plastic tubes and read on a gamma counter with specificity for ^{125}I.

It is understood that modifications made to a peptide when branched chains of chelating groups are added may alter its receptor binding capabilities. For this reason, the modified peptides were tested along with standard synthetic neurotensin as positive control. Fig. 2 shows that all the peptides competed with the radioactive traces ^{125}I-NT for binding sites. It can be seen that as the number of chelating groups on the peptide is increased, the ED_{50} in this assay shifted slightly to the right. Fig. 3 compares binding potencies calculated for neurotensin and the branched chain modified neurotensins which varied from 30-100%. As the size of the peptides increase, their binding affinity decreased, suggesting that the branched chains interfered to some extent with the binding reaction. Nevertheless, for each of the peptides, binding affinity for the receptor was sufficiently high for use in imaging.

Example 3

Quantification of Gadolinium Chelating Sites

According to the synthesis protocol, one DTPA group should be added to the peptide at each amino group. In order to verify the number of chelating groups per peptide, radioactive ^{51}Cr chromium chloride was used.

The peptide of Example 1 and ^{51}CrCl were added in the following ratios based on molar quantities of DTPA: ^{51}Cr- 1:2, 1:6, 1:8. Peptides were diluted to get 100 pmol of DTPA, and ^{51}Cr had a specific activity of 250 cpm/pmol. 50 mM sodium acetate at pH 5.5 was used to bring the reaction volume to 500 mL. The

reaction mixture was incubated at room temperature for three hours. It was found through a previous time course experiment that the reaction reached completion in three hours.

Purification was completed by using C-18 SepPak cartridges (Waters). Using a 10 cc syringe, 10 mL of methanol was passed through the sep pak to remove any air. It was then washed with 10 mL of 50 mM sodium acetate at pH 5.5 and the reaction mixture was applied slowly in 5 mL of the sodium acetate. The column was washed four times with 5 mL of 50 mM sodium acetate at pH 5.5 and the peptides were eluted with 75% acetonitrile (2 ml) into two 12x75mm tubes. The tubes were counted on a gamma counter set for ^{51}Cr efficiency.

Fig. 4 illustrates the number of counts and the ratio of ^{51}Cr to the chelating groups mol/mol. The leveling off of counts indicated that the chelating sites were saturated. These data were used to calculate the percentage of theoretically possible DTPA groups present in each peptide that were available for binding chromium (Fig. 5). The results indicate that each peptide was able to chelate an amount of chromium nearly equal to the maximum number of DTPA groups theoretically present. Thus it was found that greater than 95% of all DTPA sites were occupied by ^{51}Cr after three hours of incubation at room temperature.

These data indicated that the synthesized peptides contained the predicted amino acids and chelating elements, that they were recognized in a specific RIA towards the C-terminal region of NT and that they bound with high affinity to specific NT receptors expressed in PC3 cells.

Example 4

Animal MRI Experimentation

Adult male SD rats, weighing between 300 and 400 g were used for imaging experiments to test the contrast peptides. The rats were anesthetized by IP (intraperitoneal) injection (25 mg/kg body weight) of Nembutal. First, a small sagittal incision (10-15 mm) of skin was made over the skull, starting from the level of bregma, about 2 mm lateral to the midline. Then, a small hole with a diameter of approximately 1 mm was drilled through the skull at 1.5-2 mm caudal to the bregma, 1.8 min lateral to the midline, and 5 mm down through the dura mater. At the end of each imaging experiment, the rats were sacrificed with an overdose of Nembutal.

In order to infuse the peptides directly into the lateral ventricle during the experiment, a specially made intracranial catheter composed of silica capillary tubing (Plastics One Inc., Roanoke, VA) was used. One end of the catheter was connected to P-20 tubing, which was then fed into P-50 tubing. The total length of tubing was approximately 25 cm (long enough to reach outside of the magnet) and connected tubing segments were glued together. The end of the tubing material was connected to a 50 mL Hamilton glass syringe used for peptide injections.

Once the tubing was in place, the rat was placed in a special holder designed to keep it motionless during MRI imaging (Lahti KM, Ferris CF, Li F, Sotak CH, and King JA (1998) *Journal of Neuroscience Methods 82:* 75-83), already incorporated be reference. A plastic ear piece with ear supports designed to fit into the opening of the auditory meatus was positioned over the ears. The head was placed into the cylindrical head holder surrounded by a 5.2 cm diameter birdcage coil. The animal's canines were secured over a bite bar and its ears positioned inside the head holder with adjustable screws fitted into lateral slits. A screw over the bridge of the nose was adjusted holding the snout down onto the bite bar. The lateral screws were tightened, securing the animal's head in a fixed position. The head holder was secured to the mounting unit with plastic screws. The body of the animal was placed into the body holder and secured onto the mounting unit. During T_1 weighted imaging, 3 ml of paramagnetic NT ligand was injected into the lateral ventricle.

Fig. 6B shows paramagnetic ligand localization of putative NT binding sites (Fig. 6A) in the brain of the rat. Fig. 6A shows binding of iodinated NT to in vitro tissue slices of rat brain. The light areas show specific NT binding sites. Fig. 6B shows enhanced MR signal in comparable brain areas following the intracerebroventricular injection of 10 nM paramagnetic NT ligand.

Fig. 7 shows changes in MR signal, i.e. specific contrast, in the presence of 10 nM and 100 nM paramagnetic NT ligand in the areas of the rat brain with known high concentrations of NT receptors, including the retrosplenial cortex (RSg/Rsa), pre and para subiculum (Prs), entorhinal cortex (Ent), substantia nigra and ventral tegmental area (SN/VTA). The absence of MR signal in areas with no NT binding includes deep mesencephalic nucleus and central tegmental tract (DpMe/Ctg).

These data show the newly developed paramagnetic NT ligand can be

used to visualize receptors in the brain of fully conscious rats with high field MRI.

Example 5

Time Elapsed Binding

The cell binding studies above indicate that each peptide was able to compete with the radioactive NT and therefore bind to the appropriate receptor. As shown in Fig. 2, the peptides designed for imaging exhibited a slightly decreased binding ability. As the extent of branching increased, the ability of the peptides to bind the receptor decreased. Still, while largest peptide exhibited the lowest binding ability, it is a good candidate for imaging of receptors. The binding potency was decreased to 30%, but the eight fold increase Gd-content would be expected to off-set the slight decrease in receptor binding. It may be possible to avoid this problem by creating a longer spacer of amino acids between the receptor binding sequence and the branching arms of the gadolinium chelators.

Peptides chelated to one or several gadolinium ions can be used over time to increase imaging potential. Figs. 8C-D show signal contrast at 15 and 60 minutes post-injection of 10 μM gadolinium. Paramagnetic NT coupled to four Gd chelates was used. The data were obtained by baseline subtraction, with Figs. 8A-B showing 15 and 60 minutes post-injection of 40 μM gadolinium control. Figs. 8A-D show that the amount of signal increases for at least an hour, indicating probable internalization of the peptide-gadolinium complex.

A PC-3 cell binding assay was performed to measure total binding and internalization of chelated peptides with and without 1-4 Gds. Figs. 9 and 10 show that the chelate with 4 Gd (chelate-4-Gd) provides excellent binding. Inside binding (internalization) was measured by washing the cells and repeat measuring. Fig. 11 shows that the chelate with 4 Gd provides excellent inside binding, with approximately 75% internalization. Thus, the chelate with 4 Gd provides excellent signal optimization.

Example 6

Increased Binding Assay

To optimize binding further, the chelate with chelate-4-Gd was studied in the presence of various drugs, particularly calcium channel blockers. Chelate-4-Gd

was labeled with ^{125}I and its binding ability was measured in the presence of the drugs. Binding without drugs was assessed by using DMSO and competing with 1μM NT. All channel blockers studied, including DMSO, Nifedipine (L-type Ca^{++} channel blocker), MK886 (leukotriene inhibitor), CGS (adenosine A1 agonist), Transferrin (Fe carrier molecule), aided in internalization of chelate-4-Gd (Figs. 12-13). Other compounds that increase uptake of the peptide may be used within the scope of this invention.

Example 7

Increased Stability Peptides

Neurotensin (NT, SEQ ID NO. 1) has a half-life of less than one minute *in vivo*, thereby limiting its interaction with the NT receptor and reducing its internalization. Several modified NT analogs were prepared by replacing amino acids in the parent sequence by non-naturally occurring amino acids (wherein "non-naturally occurring" means compounds other than generally translated amino acids); modified peptides were purchased from New England Peptides, Inc. (Fitchburg, MA). Modifications focused on the stabilization of these analogs to enzymatic degradation without substantially reducing binding and internalization. While non-naturally occurring amino acids are used in this example, it will be understood that in some pathways, it may be possible to substitute other naturally occurring amino acids, particularly amino acids having similar hydrophobicity. Particular stabilization of the scissile Arg(8)-Arg(9) peptide bond, accomplished via substitution of Arg(8) with 4-piperidylalanine (Pipala), afforded a 4-fold increase in stability while retaining 35% of the binding (SEQ ID NO. 4). Replacement of either Ile(12) or Leu(13) with amino acids chosen from (S)-*tert*-butylglycine (Tle), (S)-cyclohexylglycine (Chg), or cyclohexylalanine (Cha) afforded a 3-fold increase in stability relative to NT. The following stabilized analogs were prepared:

Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Tle-Chg (SEQ ID NO. 7)

Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Tle-Leu (SEQ ID NO. 8)

Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Chg-Chg (SEQ ID NO. 9)

Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Cha (SEQ ID NO. 10)

Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Cha-Cha (SEQ ID NO. 11)

Binding was completely maintained upon replacement of Pro(10) by (S)-4-

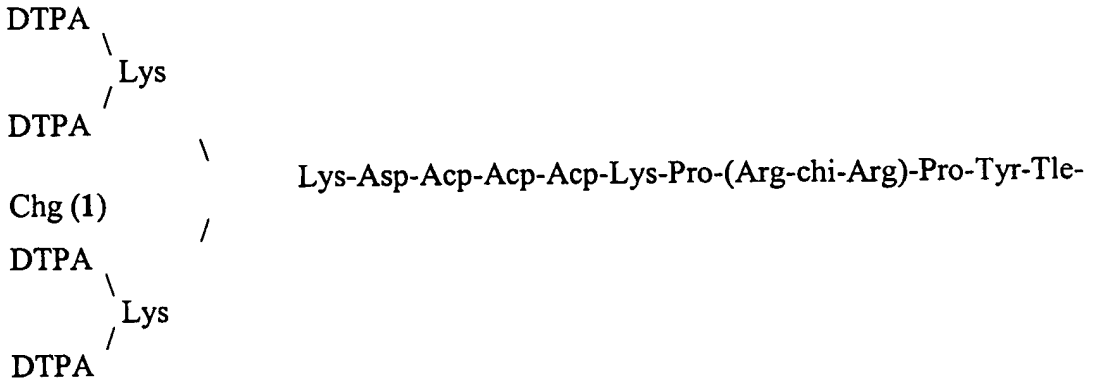
thiazolidinecarboxylic acid (Thz) (SEQ ID NO. 5) and largely preserved at 35% relative to NT upon replacement of Tyr(11) by *para*-aminophenylalanine (pNH2F) (SEQ ID NO. 6).

Hybrid NT ligands were prepared by coupling analogs of NT(7-13) to polymers of 6-aminocaproic acid (Acp). These analogs also employed the stabilizing replacements of Arg(8) by Pipala and Ile(12) by Tle. In addition, Pro(10) was optionally replaced by Thz. The hybrid ligands were subsequently coupled to the metal chelation element diethylenetriaminepentaacetic acid (DTPA) to produce the following analogs:

DTPA-Acp-Acp-Acp-Pro-Pipala-Arg-Thz-Tyr-Tle-Leu (SEQ ID NO. 12)

DTPA-Acp-Acp-Acp-Pro-Pipala-Arg-Pro-Tyr-Tle-Leu (SEQ ID NO. 13)

Hybrid NT ligands containing an expanded number of DTPA chelation elements were prepared in a similar manner. Analogs of NT(6-13) (residues 6-13 of SEQ ID NO. 1) were prepared in which the Arg(8)-Arg(9) scissile bond was blocked from proteolysis via reduction of the backbone amide to obtain the ethylenediamine dipeptide isostere Arg-chi-Arg. In addition, the scissile Ile(12)-Leu(13) peptide bond was stabilized as above by replacing Ile(12) with Tle, and optionally replacing Leu(13) with either Chg or Cha. These analogs were further converted into hybrids via sequential coupling to Acp polymer and subsequent coupling to the central residue of trimeric lysine. The multidentate DTPA chelation elements were attached to the remaining 4 primary amines of the lysine trimer to obtain the following hybrid multidentate ligands:



DTPA
\
Lys
/
DTPA
\
Lys-Asp-Acp-Acp-Acp-Lys-Pro-(Arg-chi-Arg)-Pro-Tyr-Tle-Leu (2)
/
DTPA
\
Lys
/
DTPA

DTPA
\
Lys
/
DTPA
\
Lys-Asp-Tyr-Acp-Acp-Acp-Lys-Pro-(Arg-chi-Arg)-Pro-Tyr-Tle-Cha (3)
/
DTPA
\
Lys
/
DTPA

Ligands 1-3 were tested for competitive binding to NTR and stability towards enzymatic degradation. All three ligands were at least 10-fold more stable than NT peptide. Ligand 3 showed binding that was comparable to NT peptide.

Stability of the modified ligands were tested in a rabbit lung incubation assay. Equimolar concentrations of NT, a chi bonded NT 8-13 that was purchased, and a novel NT with four Gd (Ligand 3) were incubated with fresh rabbit lung tissue for 80 minutes. Peptide metabolism was determined by competing the above samples with ^{125}I-NT in a PC-3 cell binding assay. As can be seen in Fig. 14, line C, the novel Gd chi-NT (Ligand 3) was able to compete off more of the ^{125}I-NT than the other competitors, indicating increased stability.

Although the invention has been described in detail with reference to preferred embodiments, variations and modifications exist within the scope and spirit of the invention as described and defined in the following claims.

CLAIMS

1. A magnetic resonance imaging enhancing agent comprising a receptor binding peptide chemically modified for enhanced in vivo stability, and covalently linked to a paramagnetic chelate.

2. The image enhancing agent of claim 1 wherein the paramagnetic chelate comprises gadolinium.

3. The image enhancing agent of claim 2 wherein the paramagnetic chelate comprises 1 to 8 gadolinium ions.

4. The image enhancing agent of claim 3 wherein the paramagnetic chelate comprises 4 gadolinium ions.

5. The image enhancing agent of claim 3 wherein the receptor binding compound is capable of being internalized by a cell having the receptor.

6. The image enhancing agent of claim 5 wherein the peptide is a neurotensin derivative.

7. The image enhancing agent of claim 6 wherein the peptide is modified by stabilization of the Arg-Arg bond.

8. The image enhancing agent of claim 6 wherein an Arg residue is replaced with a non-naturally occurring amino acid.

9. The image enhancing agent of claim 6 wherein the peptide is modified by replacement of the Ile or Leu residues with non-naturally occurring amino acids.

10. A method of imaging by MRI tissues having predetermined cellular receptors, said method comprising

administering to an animal having said tissues an effective amount of an imaging agent comprising a receptor-binding compound covalently bound to a paramagnetic chelate, and

monitoring MRI data for a period of time sufficient for cellular uptake of said imaging agent to obtain enhanced MRI signal intensity.

11. The method of claim 10 wherein the compound is a peptide or peptide derivative.

12. The method of claim 11 wherein the compound is modified for

enhanced stability.

13. The method of claim 11 wherein the monitoring takes place for at least sixty minutes.

14. The method of claim 11 wherein the tissues include brain cells, and the imaging agent is administered to the brain of an awake animal.

15. The method of claim 10 wherein the imaging agent is a derivative of a naturally occurring peptide capable of specific binding with said cellular receptors.

16. The method of claim 10 wherein the imaging agent is co-administered with a calcium channel blocker.

17. A composition for use in MRI comprising

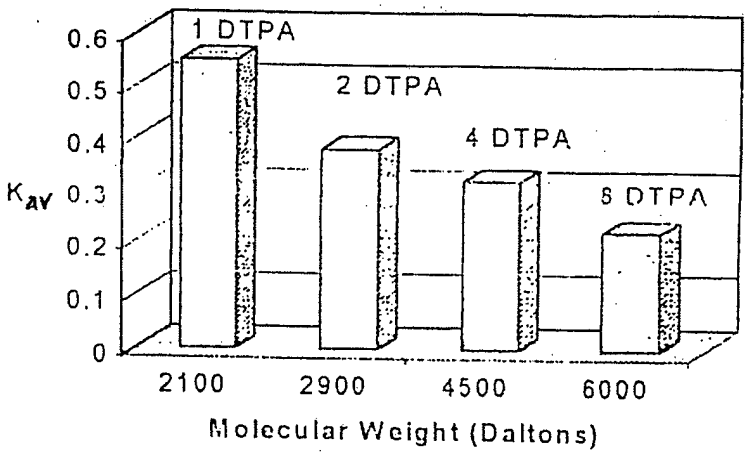
a receptor-binding compound covalently linked to a paramagnetic chelate,

a second compound that increases internalization of the receptor-binding compound, and

a pharmaceutically acceptable carrier.

18. The composition of claim 17 wherein the receptor-binding compound is a peptide or peptide derivative modified for enhanced stability.

19. The composition of claim 18 wherein the receptor-binding compound is neurotensin or a neurotensin derivative and the second compound is a calcium channel blocker.


1 DTPA
2 DTPA
4 DTPA
8 DTPA
K_{av}
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2100
2900
4500
6000
Molecular Weight (Daltons)

FIG. 1

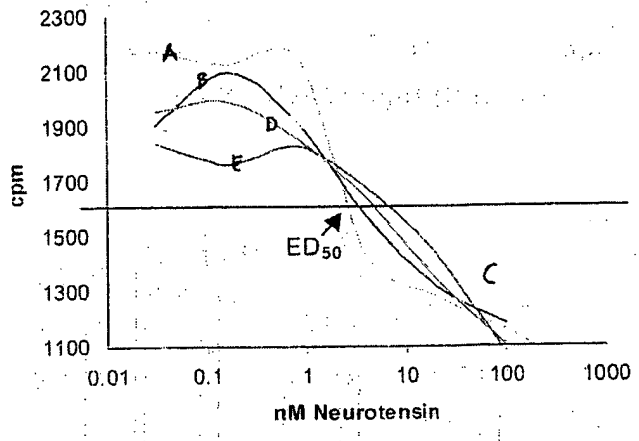

2300
2100
1900
1700
1500
1300
1100
cpm
A
B
D
E
C
ED_{50}
0.01
0.1
1
10
100
1000
nM Neurotensin

FIG. 2

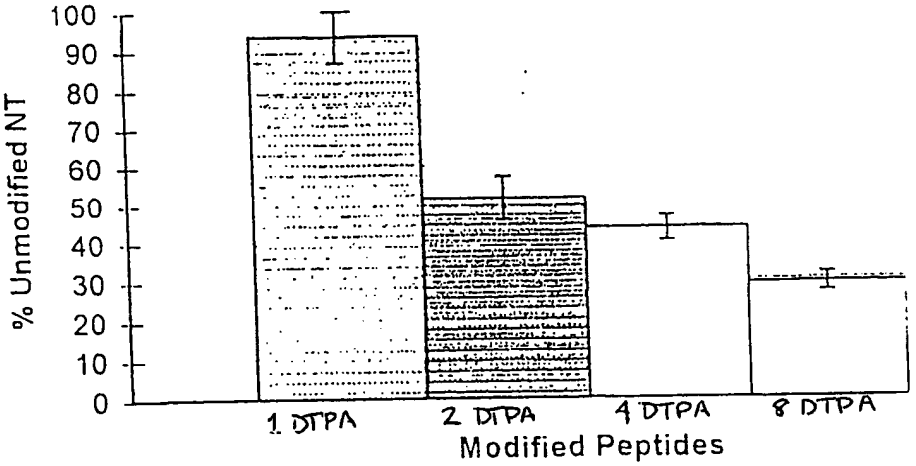


FIG. 3

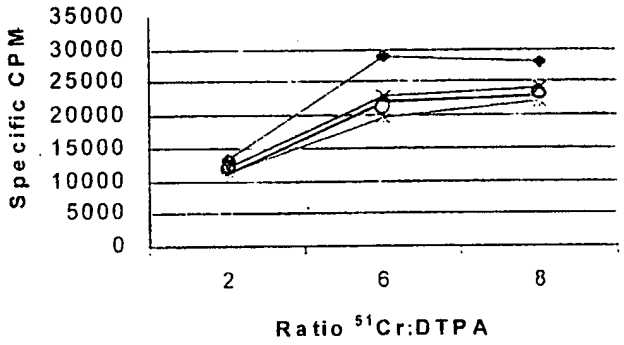


FIG. 4

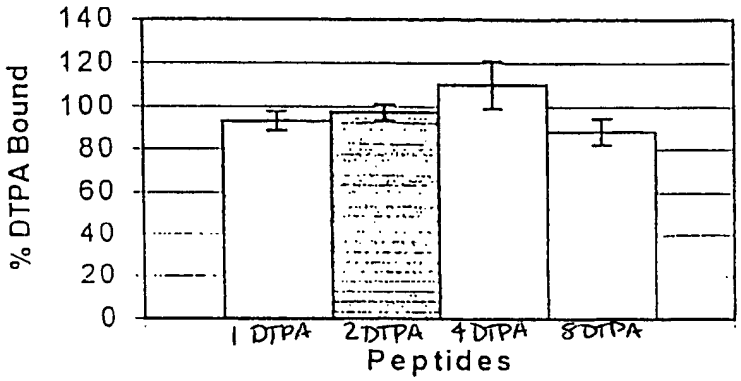


FIG 5

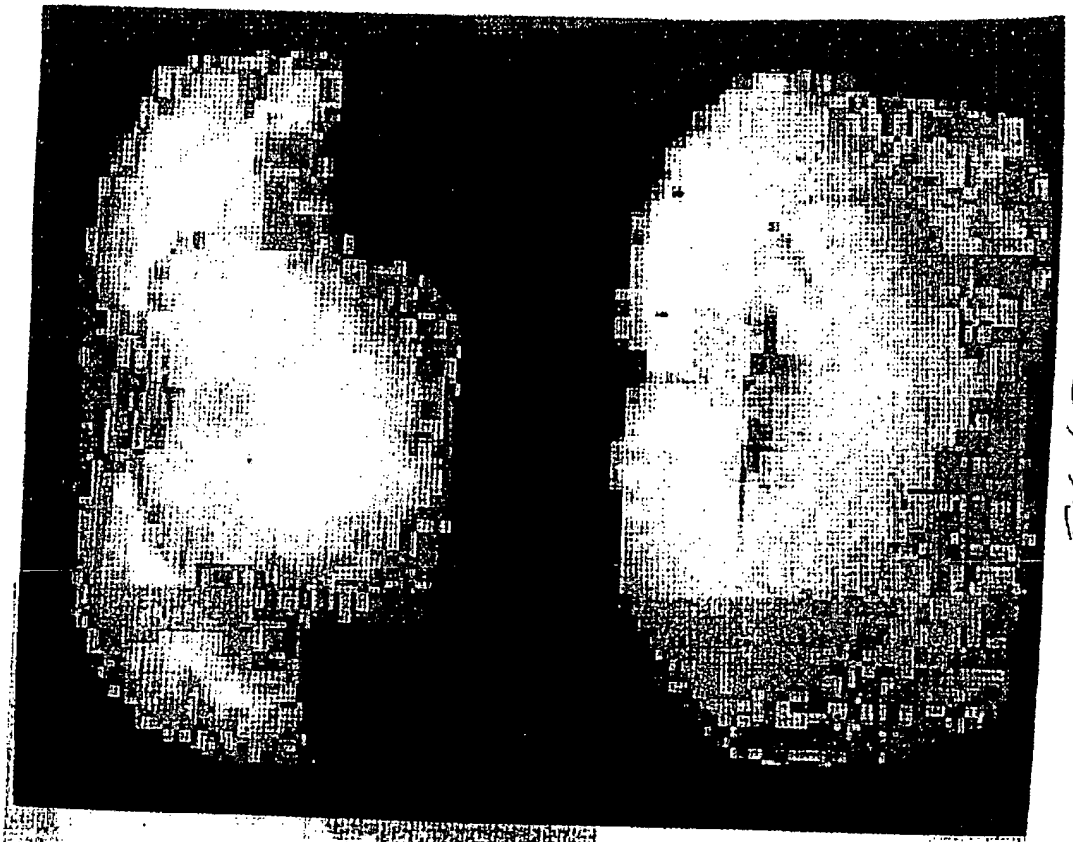


FIG 6B

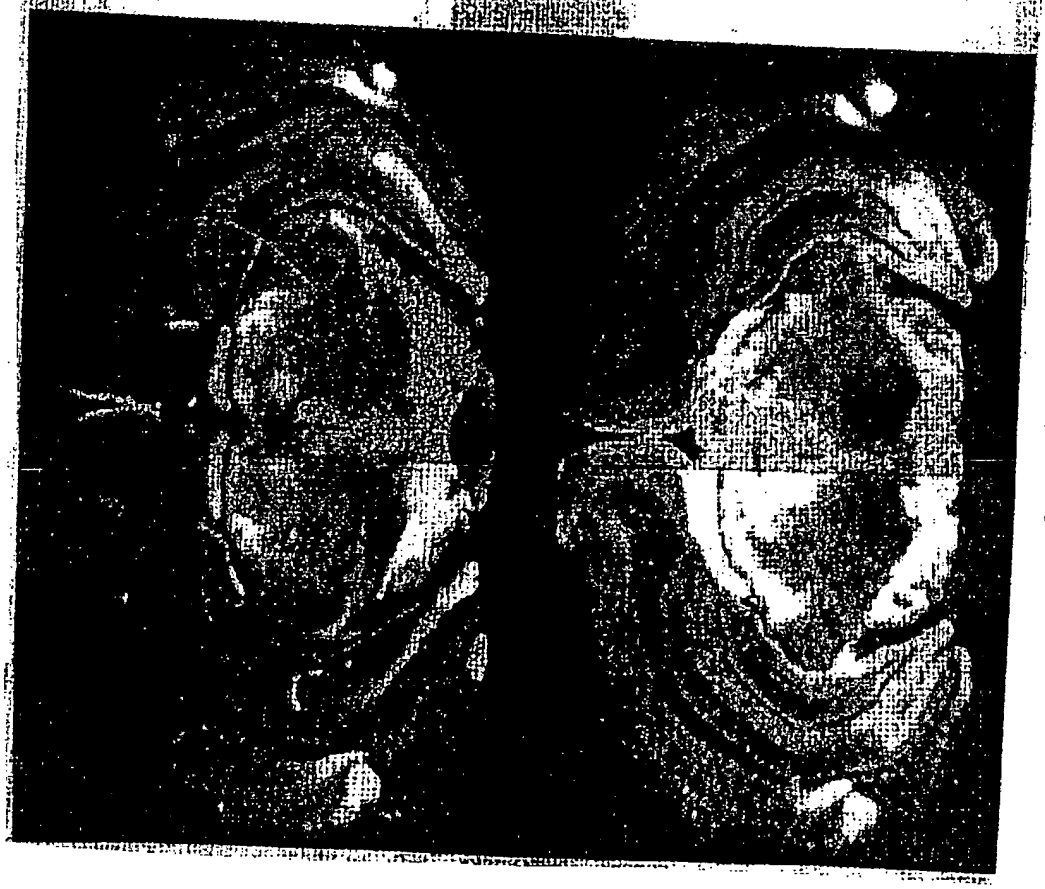


FIG 6A

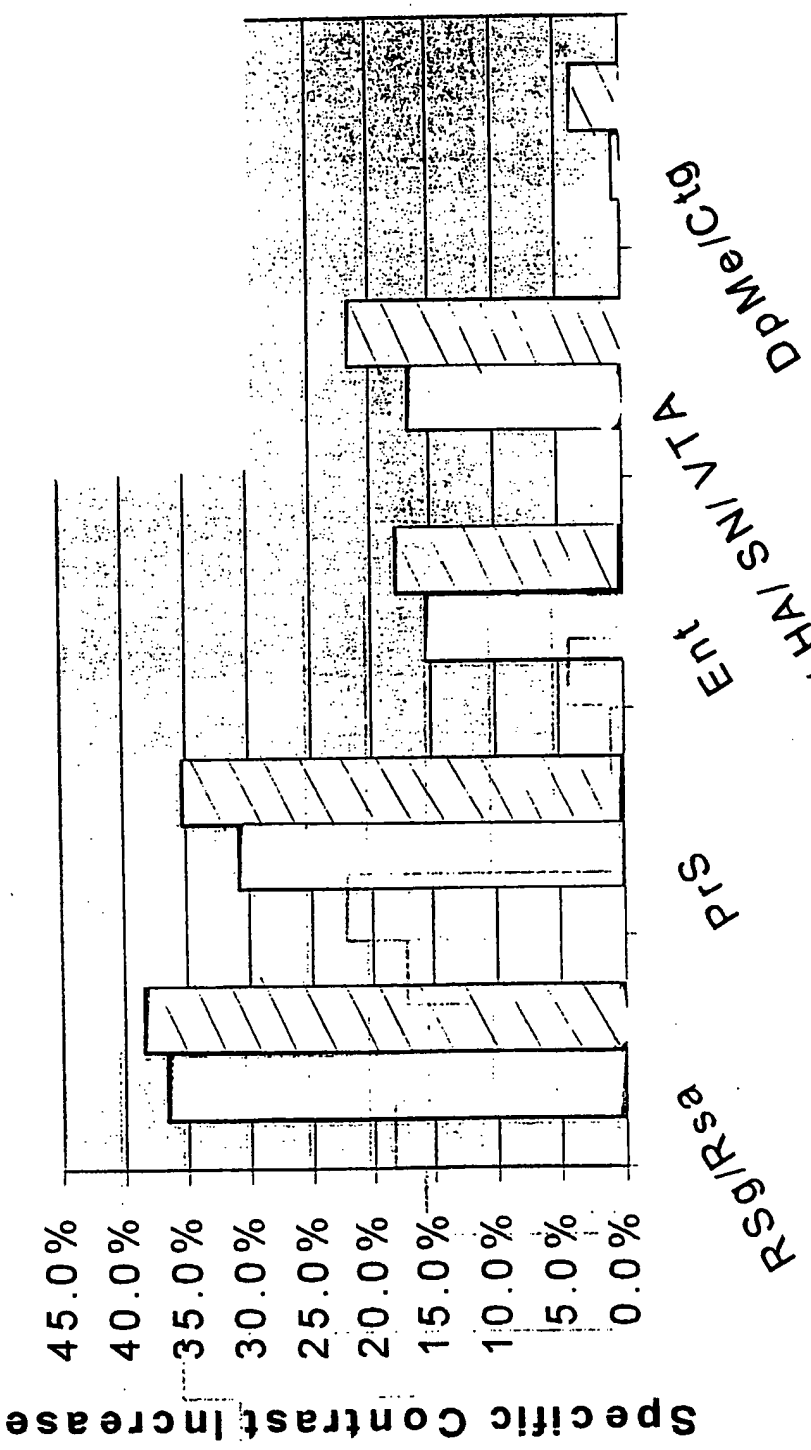

Specific Contrast Increase
45.0%
40.0%
35.0%
30.0%
25.0%
20.0%
15.0%
10.0%
5.0%
0.0%
RSg/Rsa
PrS
Ent
AAD/HAI/SNI/VTA
DPMe/Ctg

FIG. 7

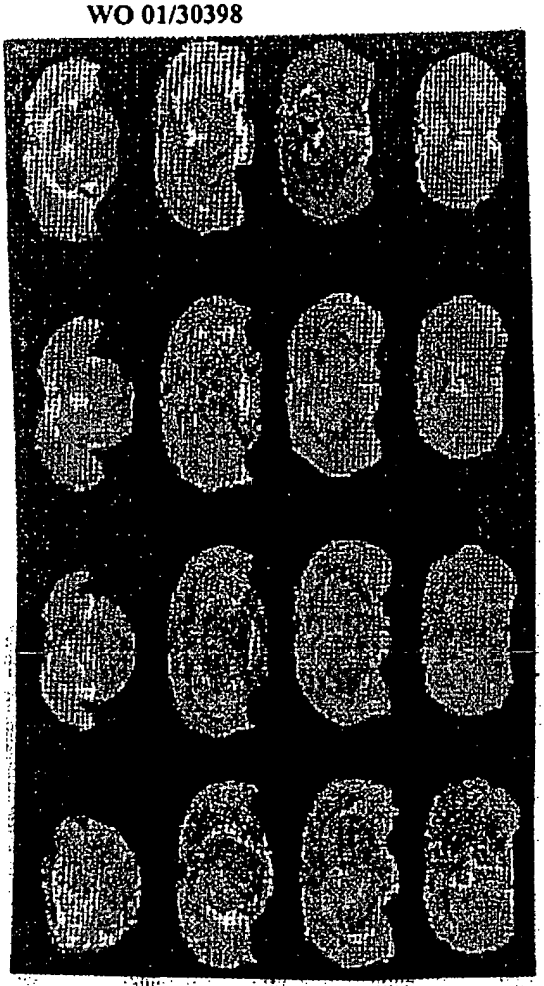


FIG. 5D

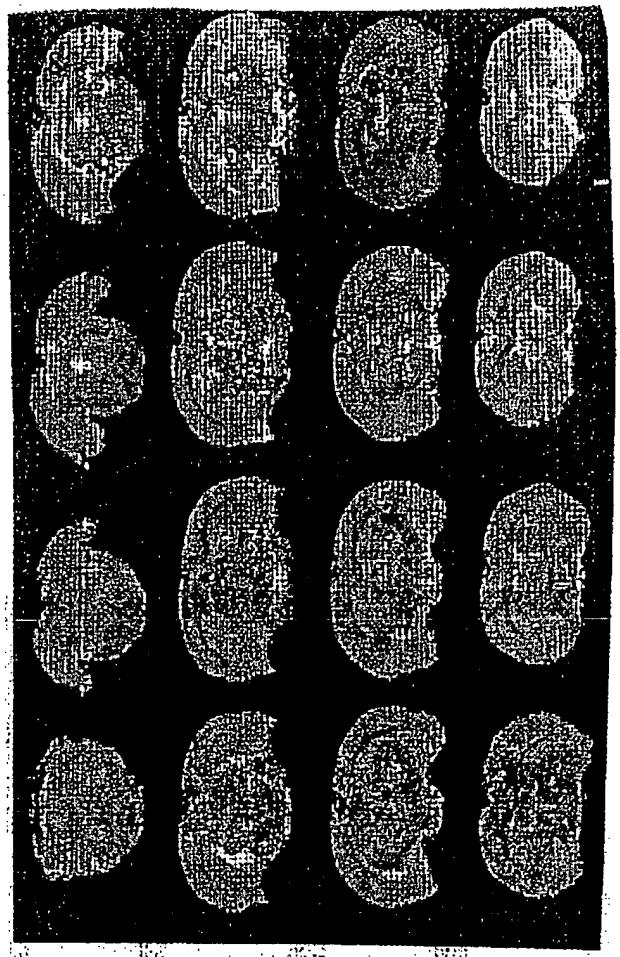


FIG. 5E

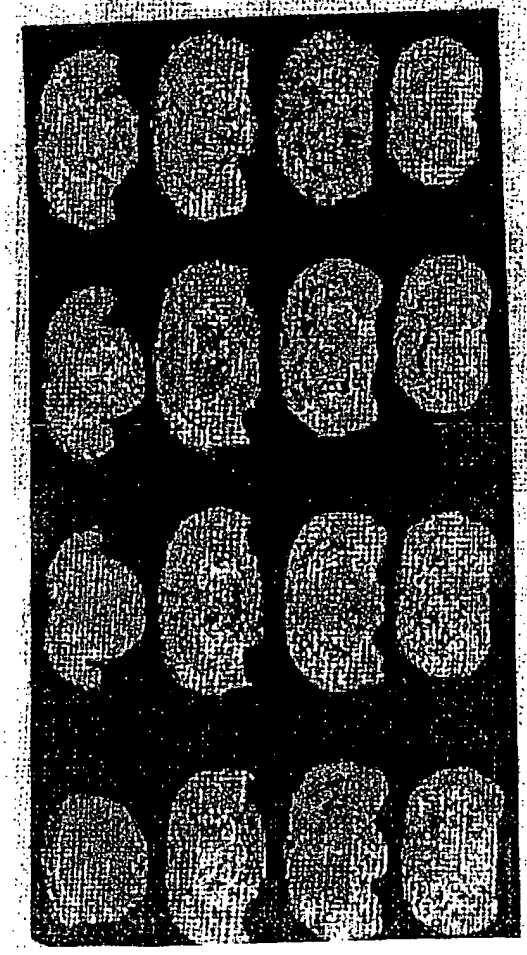
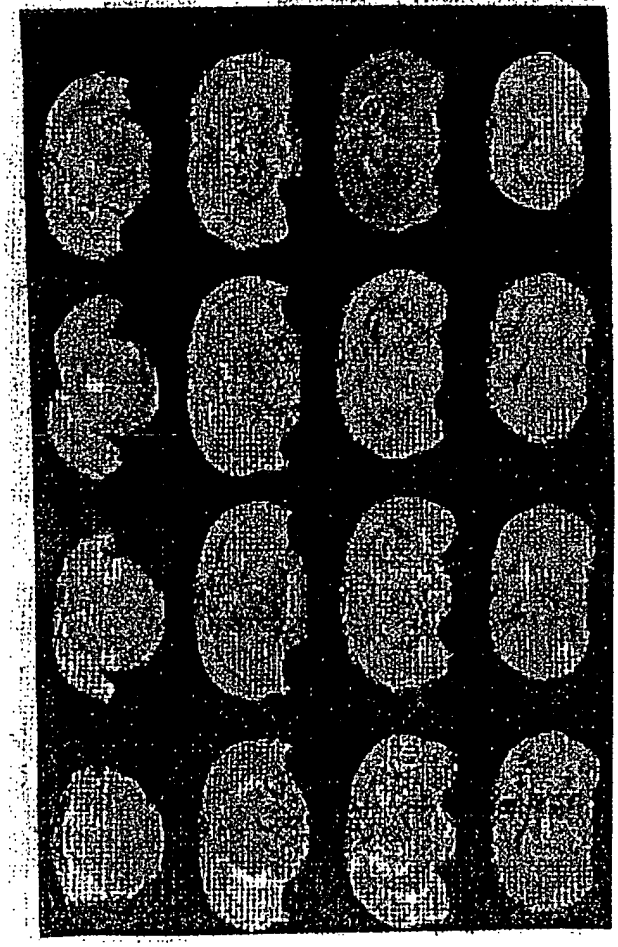


FIG. 5A



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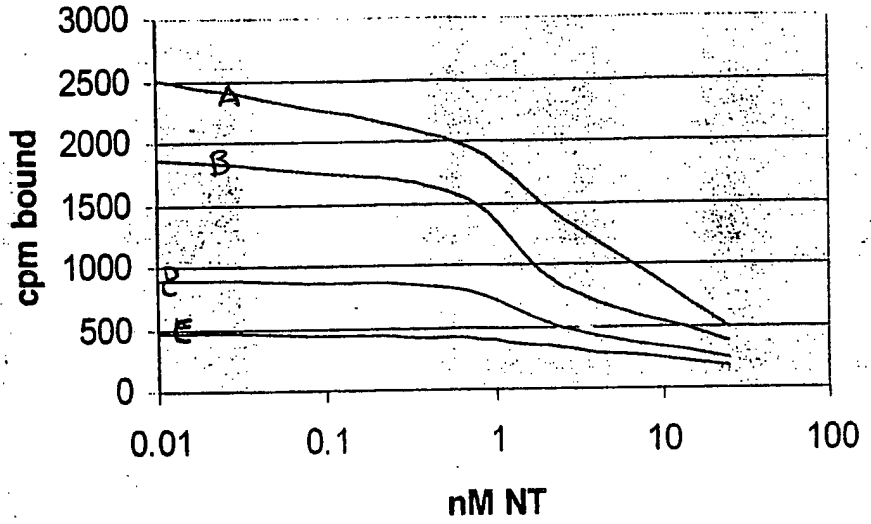
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100
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FIG. 9

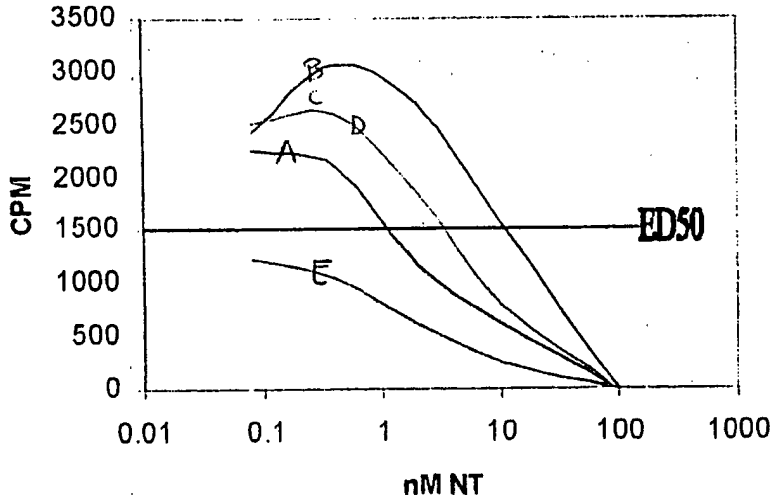
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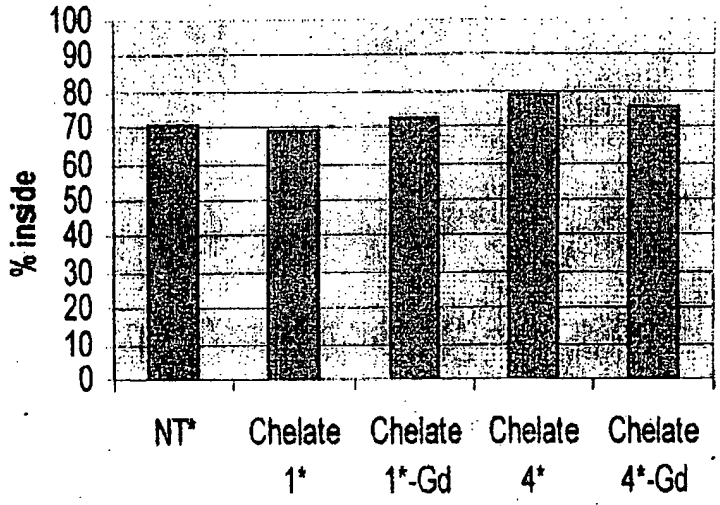
FIG. 10



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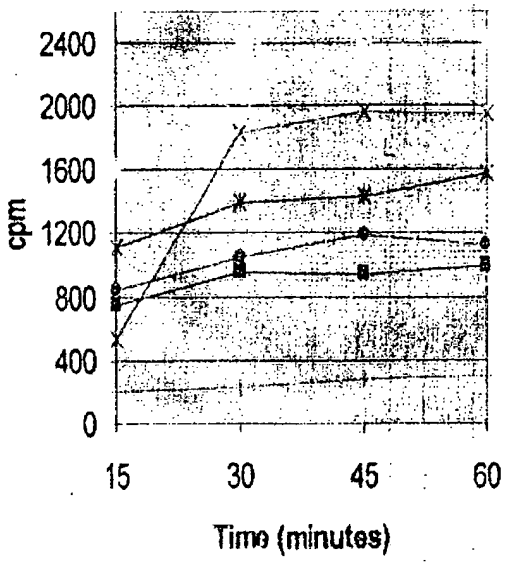

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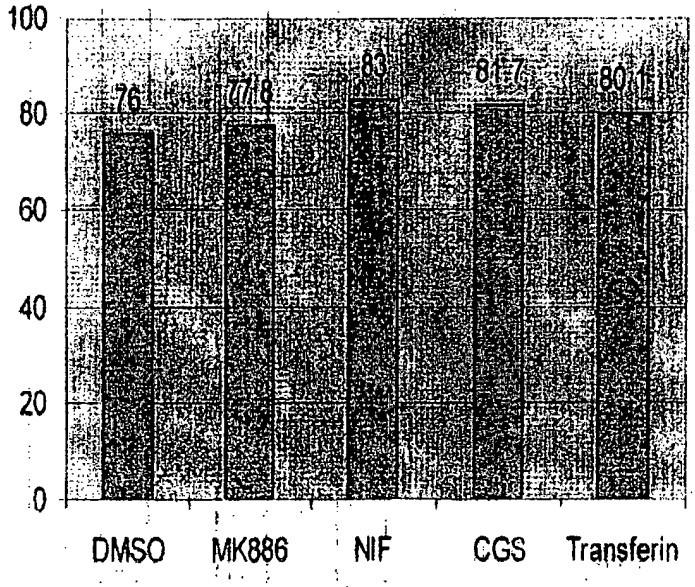

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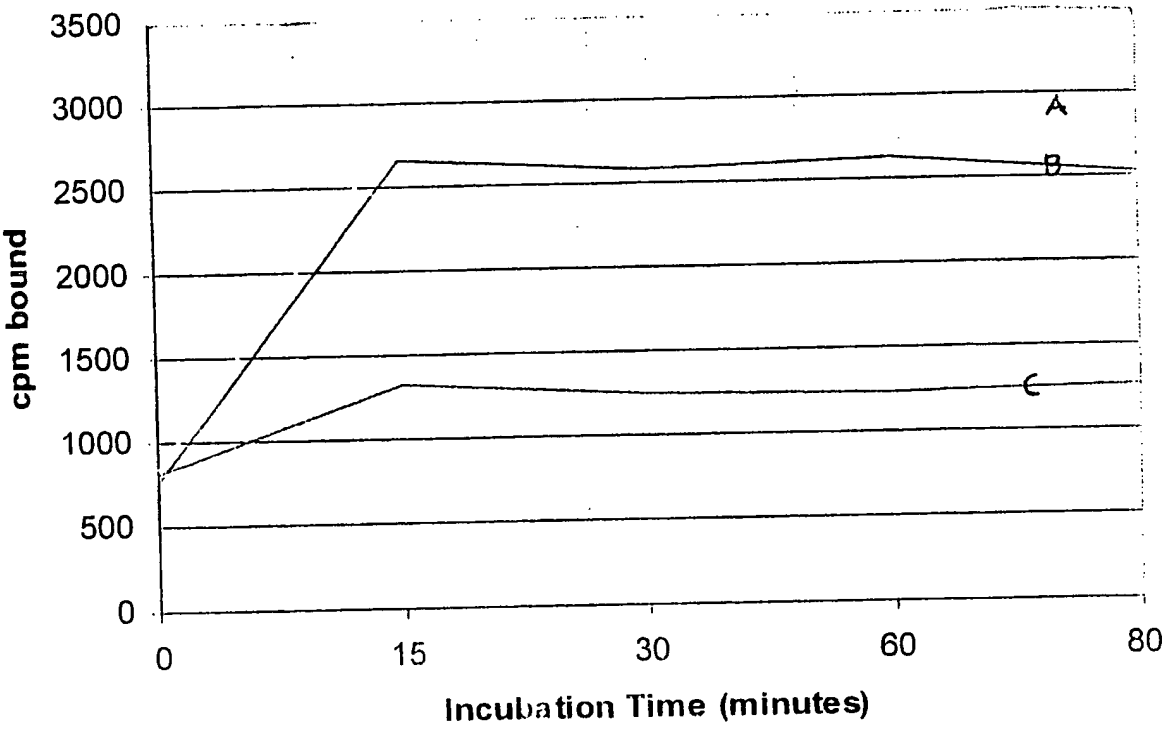
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3000
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cpm bound
0
15
30
60
80
Incubation Time (minutes)
A
B
C

FIG. 14

SEQUENCE LISTING

<110> Insight Neuroimaging Systems, LLC
Ferris, Craig
Carraway, Robert
Dudycz, Lech
King, Jean

<120> MRI Enhancing Agents Comprising Peptides

<130> 22624-67322

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(19) World Intellectual Property Organization
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(74) Agent: LAMMERT, Steven, R.; Barnes & Thornburg, 11 South Meridian Street, Indianapolis, IN 46204 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

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21 March 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/30398 A3

(54) Title: LIGAND CHELATED PARAMAGNETIC MRI CONTRAST AGENTS

(57) Abstract: Compounds and methods for imaging by MRI tissues having predetermined cellular receptors are described. The imaging agents comprise a receptor-binding ligand covalently bound to a paramagnetic chelate, optionally in combination with compound for enhancing cellular uptake of said agent. Improved MRI is achieved by acquiring MRI signals after period of time sufficient to achieve receptor mediated cellular concentration of the imaging agent.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/41385

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K49/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
CHEM ABS Data, EMBASE, BIOSIS, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO 98 33531 A (REUBI J C ;MALLINCKRODT MEDICAL INC (US)) 6 August 1998 (1998-08-06) claims	1-19
X	WO 95 22341 A (CEUSTERS MARC ;TOURWE DIRK (BE); MERTENS JOHN (BE); MALLINCKRODT M) 24 August 1995 (1995-08-24) claims	1-19
E	WO 00 78796 A (SRINIVASAN ANANTHACHARI ;ERION JACK L (US); MALLINCKRODT INC (US);) 28 December 2000 (2000-12-28) claims	1

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
27 November 2001	11/12/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Berte, M

1

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INTERNATIONAL SEARCH REPORT

Int[illegible]tional Application No
PC[illegible]/US 00/41385

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	TENNEY, J. (1) ET AL: "NMR imaging of neurotensin receptors in the brain with a newly developed paramagnetic ligand." SOCIETY FOR NEUROSCIENCE ABSTRACTS., (1999) VOL. 25, NO. 1-2, PP. 1970. MEETING INFO.: 29TH ANNUAL MEETING OF THE SOCIETY FOR NEUROSCIENCE. MIAMI BEACH, FLORIDA, USA OCTOBER 23-28, 1999 SOCIETY FOR NEUROSCIENCE. , XP001041587 abstract	1-19

INTERNATIONAL SEARCH REPORT

information on patent family members

Inte tional Application No
PCT/US 00/41385

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